# Exhibit B

"Todoroki"

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# Ropivacaine Inhibits Neurite Outgrowth in PC-12 Cells

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Peripheral nerve injury often leads to neuropathic pain, which might involve sympathetic postganglionic nerve fiber sprouting in the dorsal root ganglion. Recent studies suggest the effectiveness of ropivacaine in blocking neuropathic pain. To determine if ropivacaine affects sympathetic sprouting, we used pheochromocytoma (PC-12) cells, which differentiate into neurons on exposure to nerve growth factor (NGF). PC-12 cells were precultured in the presence of 50 ng/mL of NGF for 4 days. Neurite outgrowth was quantified as neurite extension after 24-, 48-, and 72-h exposure to ropivacaine at doses ranging from 10 to 200 μg/mL. Ropivacaine inhibited the neurite outgrowth in a dose-dependent manner. The inhibitory effect of ropivacaine was completely reversible because the NGF-stimulated neurite outgrowth was recovered to control level after washing out ropivacaine. Ropivacaine, therefore, may exert its therapeutic action on neuropathic pain, at least in part, by suppressing sympathetic sprouting.

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Neuropathic pain that follows nerve damage is often severe and persistent, even after complete recovery of damaged tissue. Sympathetic axons invade dorsal root ganglia (DRG) after nerve injury, and the activity in the resulting pericellular axonal "baskets" may underlie painful sympathetic-sensory coupling (1,2). The above-mentioned two studies published in 1993 described a novel anatomical phenomenon in axotomized rat DRG. The most economical hypothesis is that sympathetic axons form baskets in response to some factor whose bioavailability is altered in DRG after nerve injury. A likely candidate is nerve growth factor (NGF) (3,4).

A pheochromocytoma cell line (PC 12 cells) has achieved preeminence as a cellular model of sympathetic sprouting and NGF action. Zachor et al. (5) reported that cocaine irreversibly inhibited the spouting of NGF-stimulated PC-12 cells. Manivannan and Terakawa (6) reported that lidocaine inhibited the rapid sprouting of filopodia induced by electrical stimulation in chromaffin cells.

Ropivacaine is a long-acting local anesthetic that is less toxic than bupivacaine and has the additional advantage of producing less motor blockade. Several clinical and experimental reports suggest the effectiveness of ropivacaine in blocking neuropathic pain (7,8). We hypothesized that ropivacaine might reduce sympathetic sprouting, resulting in alleviation of painful peripheral neuropathy.

Because of their morphogenic response to NGF, PC-12 cells were used as a model of study concerning the direct effects of ropivacaine on the NGF-stimulated neurite extension.

## Methods

PC-12 cells (ATCC Number CRL-1721) were purchased from American Type Culture Collection (Rockville, MD), and only early passage cells were used in this study. Fetal calf serum, horse serum, and Roswell Park Memorial Institute (RPMI)-1640 were purchased from Gibco RBL (Manassas, VA). Mouse NGF (2.5 S form) was purchased from Upstate Biotech (Lake Placid, NY), and ropivacaine was from AstraZeneca Co (Osaka, Japan).

PC-12 cells were generally maintained on 100-mm tissue culture dishes at 37°C in RPMI 1640 medium, supplemented with 5% fetal calf serum and 10% horse serum, designated as serum-containing medium, in a water-saturated atmosphere of 95% air and 5% $CO_2$. The medium was changed every 3 days. For experiments, the cells were plated at a density of $10^5$ cells/mL on poly-L-lysine-coated 6-well plates or 96-well plates in serum-containing medium. After 24 h, the medium was removed, and fresh medium consisting of RPMI 1640 and 1% fetal calf serum, designated as serum-free medium (SFM), was added to each well, and NGF was added at the concentration of 50 ng/

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mL. PC-12 cells were precultured in the presence of NGF for 4 days and then exposed to ropivacaine to estimate the ropivacaine effects on NGF-stimulated neurite outgrowth and cell viability.

The incidence of definitive sprouting was estimated by morphological variables, specifically by the appearance of axodendric processes detected by phase contrast microscopy. Neurite growth was quantified as the percentage of cells bearing axodendritic processes longer than 2-cell body diameters in length. Neurites were counted in 100 cells per field in 3 separate randomly selected fields per well. Triplicate wells were used routinely for each experimental condition. Dead cells were distinguished by their phase dark appearance under phase contrast microscopy, whereas live cells appear phase bright. Dead cells were not included in the quantification. Cells in clusters were not included.

Cell viability was determined by the method of modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (9). Briefly, PC-12 cells were plated on 96-well microtiter plates, in which the MTT solution (5 mg/mL in phosphate-buffered saline) was added, and 4 h were allowed for incubation. Then, cell lysis was accomplished by 100 $\mu$L of extraction buffer (20% sodium dodecyl sulfate/50% *N*,*N*-dimethyl-formamide, pH value 4.7) per well. After incubation overnight, the absorbance at 570 nm was measured (Inter Med microplate reader). At least 4 independent experiments were performed. Results were expressed as the percentage of the absorbance of the vehicle-treated control culture wells.

Data are presented as the mean ± SD. Statistical analysis was made by analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons. A value of $P < 0.05$ was considered significant. These tests were performed using Stat View 5.0 software (SAS Institute Inc, Hulinks CO, Tokyo, Japan).

## Results

NGF exposure produced a monotonic increase in the percentage of neurite outgrowth. The average percentage of cells with neurite after 4 days' exposure to 50 ng/mL of NGF was 35% in SFM (Fig. 1).

PC-12 cells were exposed to various concentrations of ropivacaine, from 10 to 1000 $\mu$g/mL, for 3 days. Ropivacaine caused a time- and dose-dependent decrease in cell viability (Fig. 2). Ropivacaine at up to 100 $\mu$g/mL did not cause extensive cell death even after 3 days' incubation. The cell viability decreased at a large dose of ropivacaine. Cells exposed to 800 and 1000 $\mu$g/mL of ropivacaine began to die within 2 h, and 75% of cells died within 2 days.

Ropivacaine had significant dose-related inhibitory effects on NGF-stimulated PC-12 neurite extension.

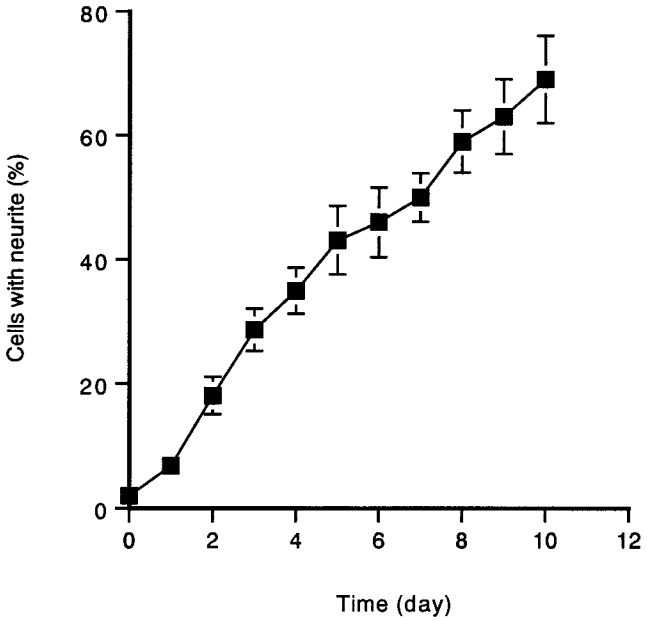


**Figure 1.** Time-response curve of nerve growth factor (NGF) induced pheochromocytoma (PC-12) cell neurite outgrowth. The cells treated with NGF at 50 ng/mL for various periods of time (horizontal axis). A neurite growth (vertical axis) was quantified as the percentage of cells bearing axodendritic processes longer than two cell diameters in length. Neurites were counted in 100 cells per field in 3 separate fields per well. Triplicate wells were used for each experimental condition. Values represent mean ± SD of the percentage of neurite outgrowth.

Incubation with 100 and 200 $\mu$g/mL of ropivacaine significantly reduced the number of cells with neurite when observed 1, 2, and 3 days after incubation with ropivacaine. Ropivacaine at 10 $\mu$g/mL had no significant inhibitory effect on neurite extension (Fig. 3). Cells incubated with 400 or 600 $\mu$g/mL of ropivacaine revealed no sprouting of their neurite throughout incubation period (data not shown).

Recovery from the inhibitory effects of ropivacaine was defined as a significant increase in the percentage of cells, demonstrating NGF-related neurite outgrowth after washing out ropivacaine. Cells were treated with NGF and ropivacaine 100 $\mu$g/mL for 3 days and were allowed to recover in NGF-containing SFM for another 3 days.

The morphologies of PC-12 cells treated with ropivacaine are demonstrated photographically in Figure 4. In cells treated with 100 $\mu$g/mL of ropivacaine, neurite growth was prevented.

## Discussion

The major finding of the current study is that ropivacaine has a direct and reversible dose-dependent inhibitory effect on NGF-stimulated PC-12 cell neurite extension without affecting cell viability. The present

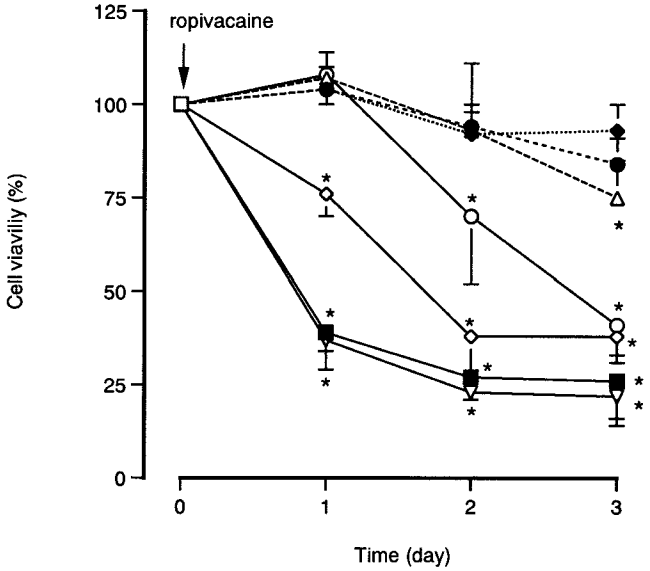


**Figure 2.** Effects of ropivacaine on cell viability. Pheochromocytoma (PC-12) cells were exposed to ropivacaine 0 μg/mL (open square), 10 μg/mL (closed diamond), 100 μg/mL (closed circle), 200 μg/mL (open triangle), 400 μg/mL (open circle), 600 μg/mL (open diamond), 800 μg/mL (closed square), or 1000 μg/mL (inverted open triangle) for 3 days. Cell viability was determined by the method of modified MTT assay. Ropivacaine doses ranging from 10 to 100 μg/mL did not affect cell viability, even after 3-day incubation. The data were presented as the mean ± SD, from at least four independent experiments in quintuplicate. Statistical analysis was made by analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons. The star symbol compares ropivacaine treatment with control values. A value of $P < 0.05$ (*) was considered significant.

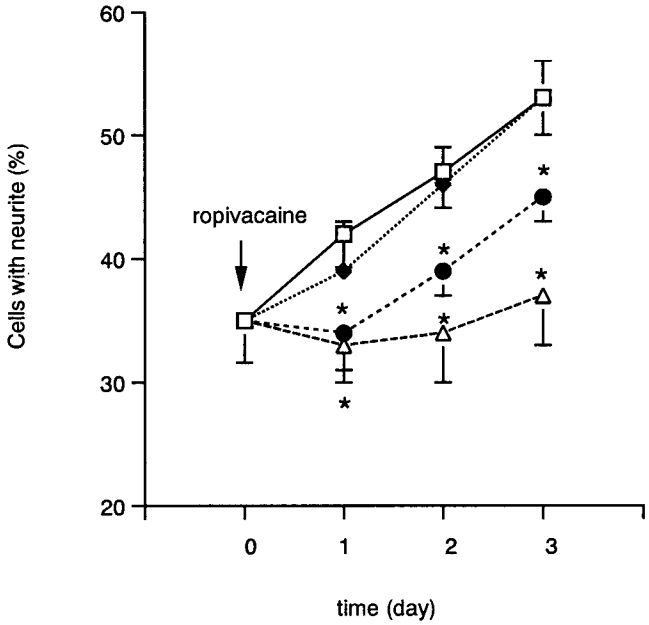


**Figure 3.** Inhibitory effects of ropivacaine on neurite outgrowth. Incubation with 100 and 200 μg/mL of ropivacaine significantly reduced the number of cells with neurite when observed 24, 48, and 72 h after treated with ropivacaine. The data were presented as the mean ± SD, from at least 3 independent experiments. Statistical analysis was made by analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons. A value of $P < 0.05$ (*) was considered significant. The marks for doses of ropivacaine exposed are the same as in Figure 2.

result showed that ropivacaine at larger concentrations more than 200 μg/mL affected cell viability. Smaller doses of ropivacaine used in this study were less toxic to cell survival. Toxic effects of local anesthetics have been reported. Tan et al. (10) reported that PC-12 cells were exposed to procaine, lidocaine, bupivacaine, or tetracaine at their clinically relevant concentration for 10 hours, and tetracaine at 1 mM induced cell death; 1 mM of other local anesthetics tested did not influence cell survival. In their case, the much larger dosage of local anesthetics was used, and PC-12 cells were not treated with NGF, which could have influenced cell viability.

In clinical studies, the total plasma levels of ropivacaine were evaluated after intravascular ropivacaine injection, in which convulsions were induced at plasma concentrations between 1.4 and 3.6 μg/mL (5.1 and 13.1 μM, respectively) (11). The plasma concentration of ropivacaine will depend on the dose and the site of administration. When 50 mg was infused over 15 minutes IV in volunteers, the maximum concentration ranged from 1.1 μg/mL to 2.0 μg/mL (12). When given epidurally, a 100-mg dose produced a maximum concentration of 0.53–0.76 μg/mL and, a 200-mg dose produced a maximum concentration of 0.93–1.53 μg/mL (13,14). Lierz et al. (7) reported that for therapy of phantom pain, an initial bolus of 20 mL of ropivacaine 0.2% was followed by an infusion of ropivacaine 0.2% at a rate of 4 mL/h for two days and at 6 mL/h for the next four days, and the ropivacaine plasma level after five days of treatment was 3.11 μg/mL, and the patient had no pain with this regimen and did not need any other analgesics. It is difficult to compare *in vitro* concentrations with tissue levels or plasma levels *in vivo*. We used ropivacaine at concentrations from 10 to 200 μg/mL, which was approximately 3–60 times the clinical blood levels.

Ramer and Bisby (15) reported that sympathetic sprouting in DRG was observed four days after a constrictive nerve injury, paralleling the genesis of neuropathic pain. To examine the effects of ropivacaine on neurite outgrowth, we used PC-12 cells treated with NGF for four days. When exposed to NGF for four days, the average percentage of cells with neurite was 35%. The present study showed that the ropivacaine effects related to duration of exposure and that neurite growth is limited beyond a specific exposure time. Ropivacaine exposure of at least one day was required to affect neurite growth measured at three days, and the effect of ropivacaine exposure was

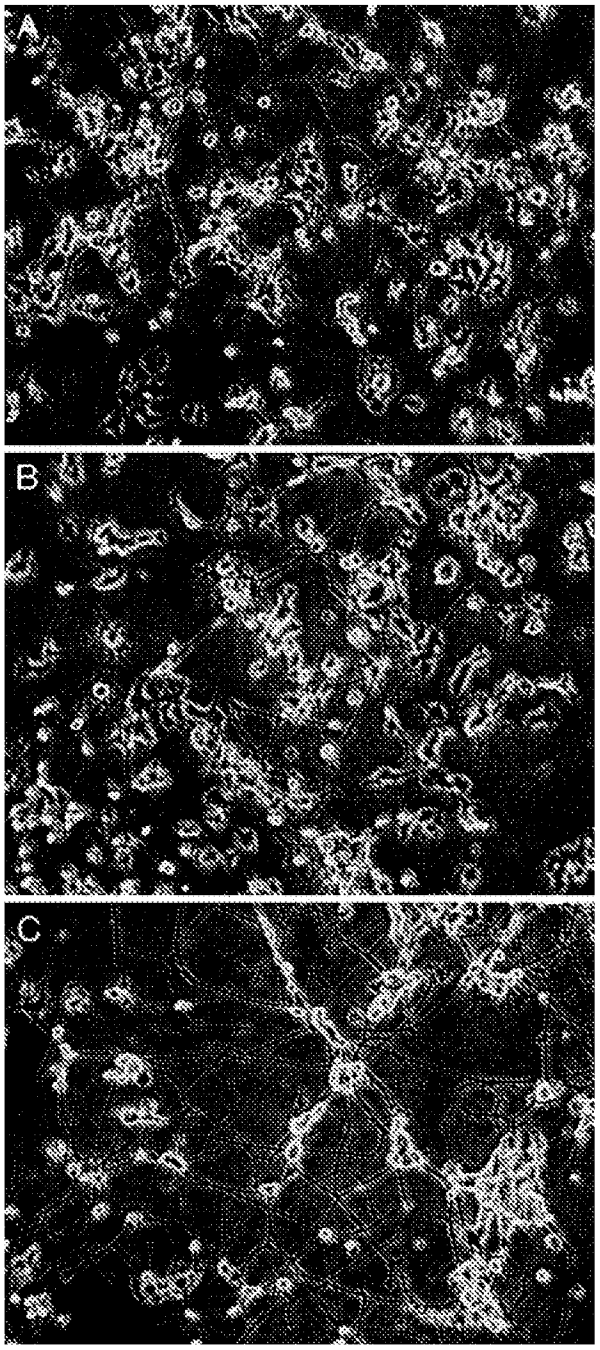


**Figure 4.** Photographs of pheochromocytoma (PC-12) cells treated with or without ropivacaine. (A) Nerve growth factor (NGF) treated PC-12 cells neurite outgrowth after 7 days' exposure to 50 ng/mL of NGF. (B) PC-12 cells exposed to NGF (50 ng/mL) for 4 days and both NGF and ropivacaine (100 μg/mL) for another 3 days. Note the significant reduction of neurite extension. (C) Cells were treated with ropivacaine (100 μg/mL) for 3 days and were allowed to recover in NGF-containing medium for another 3 days. Further neurite extension (100% of controls) was noted.

maximal at one day (75% of control cells); greater inhibition was not achieved by longer exposure. Significant recovery was noted in cells re-exposed to NGF without ropivacaine.

Sympathetic basket-skeins were observed in DRGs removed from neuropathic pain patients, but the functional relation between these structures and sensory symptoms remains speculative.

The signal responsible for this sympathetic sprouting is unclear. There is evidence that NGF may be involved (15–17). The mechanism by which NGF induces sympathetic sprouting is not clear, but many steps have been uncovered. NGF binds tyrosine kinase receptor (trk [140]) and activates an intracellular signaling cascade leading to production of new protein synthesis (18). NGF induces sustained activation of classical mitogen-activated protein kinase (also known as extracellular signal-regulated kinase [ERK]) and neuronal differentiation in PC-12 cells. In PC-12 cells, NGF rapidly stimulates the ERKs. Kinases activation is sustained and followed by the translocation of ERK 1 and ERK 2 into the nucleus, ultimately leading to neurite outgrowth (19). Ropivacaine may affect one or many steps of this signaling cascade. However, the cellular mechanisms underlying the ropivacaine-induced inhibition of neurite growth are not known.

In conclusion, ropivacaine has an inhibitory effect on neurite outgrowth of PC-12 cells in a dose-dependent manner. Ropivacaine, therefore, may exert its therapeutic action on neuropathic pain, at least in part, by suppressing sympathetic sprouting.

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